(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 359 161 A2

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
05.11.2003 Bulletin 2003/45

(51) Int Cl.[7]: **C07K 16/40**, C12N 5/20, G01N 33/577

(21) Application number: **03008868.6**

(22) Date of filing: **29.04.2003**

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LI LU MC NL PT RO SE SI SK TR
Designated Extension States:
AL LT LV MK

(30) Priority: **30.04.2002 JP 2002128312**

(71) Applicant: **NITTO BOSEKI CO., LTD.**
Fukushima-shi (JP)

(72) Inventors:
- **Ohashi, Tatsuya, Baiokemikaru Kenkyusho Fukuhara, Koriyama-shi, Fukushima (JP)**
- **Miura, Toshihide, Baiokemikaru Kenkyusho Fukuhara, Koriyama-shi, Fukushima (JP)**
- **Igarashi, Yoshihiko, c/o Dokkyo Univ.Sch.Medicine Mibumachi, Shimotsuga-gun, Tochigi-ken (JP)**
- **Sasagawa, Kumiko, Baiokemikaru Kenkyusho Fukuhara, Koriyama-shi, Fukushima (JP)**
- **Katayama, Katsuhiro, c/o Nitto Boseki Co., Ltd. Koriyama-shi, Fukushima (JP)**

(74) Representative: **HOFFMANN - EITLE**
Patent- und Rechtsanwälte
Arabellastrasse 4
81925 München (DE)

(54) Monoclonal antibody specific to tartrate-resistant acid phosphatase 5b and use thereof

(57) Monocolonal antibodies having a higher reactivity with tartrate-resistant acid phosphatase 5b (TRACP 5b) than tartrate-resistant acid phosphatase 5a (TRACP 5a) and having a higher specificity to TRACP 5b can be obtained by cell fusion using as antigens TRACP 5b purified from human osteoclasts. By using the monoclonal antibody, TRACP 5b in a sample can be detected specifically with a high sensitivity.

EP 1 359 161 A2

Description

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to a monoclonal antibody specific to tartrate-resistant acid phosphatase 5b (TRACP 5b: otherwise known as osteoclast-derived tartrate-resistant acid phosphatase), hybridomas capable of producing the monoclonal antibody, a method of detecting TRACP 5b using the monoclonal antibody, and a kit for use in the detecting method.

[0002] The monoclonal antibody of the present invention enables to specifically assay the activity of tartrate-resistant acid phosphatase 5b and is extremely useful as a marker for bone resorption in the field of medical treatments for bone diseases and clinical diagnosis.

Description of the Related Arts

[0003] It is reported that tartrate-resistant acid phosphatase (TRACP: Tartrate-Resistant Acid Phosphatase EC 3.1.3.2) in serum is an acid phosphatase derived largely from osteoclasts and that the assay of TRACP activity will be useful as measures for evaluating the function of osteoclasts. Thus, a keen attention was drawn to TRACP as a marker for bone resorption (KOTSU TAISHA MARKER (Bone Metabolism Marker), edited by Masao Fukunaga, Toshitaka Nakamura & Toshio Matsumoto, 1995, published by Medical Review, Inc.) Analysis of acid phosphatases in serum on polyacrylamide gel electrophoresis has identified 6 bands, which are Bands 0 to 6 from the starting point. In these 6 bands, Band 5 is resistant to tartrate treatment and therefore called Band 5 tartrate-resistant acid phosphatase (TRACP 5). TRACP 5 is further separated into components located at bands 5a and 5b by their electrophoretic mobility, i.e., band 5a abundant in sialic acid-binding carbohydrate chains and band 5b having less sialic acid-binding carbohydrate chains. The TRACP 5a enzyme originating from platelets, etc. does not change its blood level but only TRACP 5b changes the blood level accompanied by bone resorption. Therefore, TRACP 5b is considered to be the only one acid phosphatase derived from osteoclasts that is resistant to inhibition by tartrate (JP2002-510050A).

[0004] Abbreviation of TRACP 5b to osteoclast-derived ACP is recommended also in Clin. Chem. 47:1497, 2001. Accordingly, throughout the specification, TRACP 5b is used to mean ACP derived from osteoclasts as a marker for bone resorption and osteoclast-derived tartrate-resistant acid phosphatase and tartrate-resistant acid phosphatase 5b are collectively referred to as TRACP 5b, unless otherwise indicated.

[0005] Conventional methods of assaying TRACP activity as a biomarker of acid phosphatases to determine osteoclastic activity encounter problems in specificity, sensitivity, complicated measurements and measuring time.

[0006] In general, the assay of TRAP 5b activity can be performed by using a phosphoric acid ester as a synthetic substrate in the presence of tartrate and colorimetrically measuring the end product (alcohol or phenol) produced by the enzymatic reaction. In this assay, the tartarate inhibits prostate-derived acid phosphatase. Thus, the activity of acid phosphatase remained is measured on the substrate and then, the TRACP activity calculated from the measurements is regarded to be the TRACP 5b activity. However, this method is not very specific, since tartrate inhibits other acid phosphatases originating from erythrocytes and platelets present in serum samples, since the method includes these other acid phosphatases, in addition to the osteoclast-derived acid phosphatase. In order to improve the method above, it was proposed to pretreat serum by incubation of its 5-fold dilution at 37°C for an hour and then measure the TRACP activity in the presence of tartrate using p-nitrophenyl phosphate (pNPP) as a substrate (NICHIDAI-ISHI, 49: 904-911, 1990; Clin. Chem., 33: 458-462, 1987). This improved method can exclude acid phosphatases derived from erythrocytes but is affected by platelet-derived acid phosphatases. For a more specific method for assaying the activity, the present inventors previously found that there is a difference in resistance to a fluoride between TRACP 5b and erythrocyte- and platelet-derived acid phosphatases, and reported a method for determination of TRACP 5b based on the difference in resistance (JP 10-37198A). This method could eliminate any influence of erythrocyte- and platelet-derived tartrate-resistant acid phosphatases but was still affected by TRACP 5a. Besides, the method requires to determine the TRACP 5b activity by measuring the total tartrate-resistant acid phosphatase activity, and then calculating the difference between the total activity and the activity not inhibited in the presence of a fluoride. Therefore, a further improvement is desired in view of sensitivity. Another method reported involves use of an additional TRACP 5b inhibitor in the aforesaid method using a fluoride to determine the TRACP 5b activity more specifically (JP 2001-231595A). The method using a fluoride alone is more specific but a problem still remains in accuracy because the osteoclast-derived TRACP 5b activity is assayed based on the difference calculated, as in other known methods.

[0007] On the other hand, the following methods using a polyclonal antibody or a monoclonal antibody are also known for immunoassay methods to assay the TRACP 5b activity (J. Clin. Endocrinol. Metab., 71: 442-451, 1990; J. Bone Miner. Res., 13: 683-687, 1998; Immunol. Lett., 70: 143-149, 1999; J. Bone Miner. Res., 14: 464-469, 1999; Clin.

Chem., 45: 2150-2157, 1999; Clin. Chem., 46: 1751-1754, 2000). In these methods, an influence of TRACP 5a is not negligible because TRACP 5a and TRACP 5b are both measured undesirably without discriminating from one another. An immunoassay for measuring TRACP 5b more specifically is reported also in WO 99/50662 and JP2002-510050A. While this method is more specific to the TRACP 5b activity, the antibody used for the immunoassay is not specific to TRACP 5b and is also reactive with TRACP 5a. Then the TRACP 5b activity should be assayed by calculation from the measurement data in the immunoassay, taking advantage of the difference in optimum pH between TRAP 5b and TRAP 5a. For this reason, it is concerned to affect specimens from patients with terminal renal failure showing an increased TRACP 5a level, leading to inaccurate assay data. Furthermore, the difference between normal specimens and pathologic specimens with accelerated bone resorption is so small that the sensitivity required for a bone resorption marker is not obtained (Clin. Chim. Acta, 301: 147-158, 2000).

SUMMARY OF THE INVENTION

[0008] In view of the foregoing problems, the present invention aims at providing a monoclonal antibody specific to an osteoclast-derived tartrate-resistant acid phosphatase (TRACP 5b) well functioning as a marker for bone resorption, a hybridoma capable of producing the monoclonal antibody, a method of detecting TRACP 5b using the monoclonal antibody and a kit for use in the method.

[0009] The present invention relates to a monoclonal antibody to TRACP 5b, which provides a higher reactivity with tartrate-resistant acid phosphatase 5b (TRACP 5b, also called osteoclast-derived tartrate-resistant acid phosphatase) than that with tartrate-resistant acid phosphatase 5a (TRACP 5a) and has a higher specificity to TRACP 5b.

[0010] The present invention further relates to a hybridoma capable of producing the monoclonal antibody described above.

[0011] Furthermore, the present invention relates to a method of detecting TRACP 5b, which comprises detecting TRACP 5b according to an immunoassay using the monoclonal antibody described above.

[0012] Still further, the present invention relates to a kit for use in the detecting method, comprising the monoclonal antibody described above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013]

FIG. 1 shows the results of analysis of TRACP 5b isolated and purified from human femoral caput by SDS-PAGE.
FIG. 2 shows the results of analysis of TRACP 5a and TRACP 5b purified from human cord blood by disc electrophoresis.
FIG. 3 shows the results of analysis of recombinant TRACP produced from host insect cells by SDS-PAGE.
FIG. 4 shows the results of analysis for the reactivity of anti-TRACP monoclonal antibody 9C5 used as a positive control with human TRACP 5a, TRACP 5b and recombinant TRACP, by western blotting.
FIG. 5 shows the results of microscopic observation after reacting monoclonal antibody Trk62 of the invention with a frozen section of human osteoclast tumor tissue.

DESCRIPTION OF THE PREFERRED EMBODIEMNTS

[0014] The monoclonal antibody of the invention can be acquired by using as an immunogen TRACP 5b purified from human osteoclast cells. In the embodiments described below, TRACP 5b purified from normal osteoclast cells was used as an immunogen for immunization; not only the purified TRACP 5b but TRACP 5b from osteoclastic tumor may also be used as an antigen.

[0015] The monoclonal antibody of the invention can be produced from the hybridoma acquired by immunizing an animal using, e.g., purified human TRACP 5b as an antigen and fusing anti-human TRACP 5b antibody-producing cells produced in the animal to myeloma cells.

[0016] The hybridoma can be produced by the following procedures. That is, human TRACP 5b acquired as described above is mixed with known adjuvant such as Freund's complete or incomplete adjuvant, aluminum hydroxide adjuvant, pertussis adjuvant, etc. to prepare an adjuvant solution for sensitization, which is administered to animal such as mice or rats, intraperitoneally, subcutaneously or via the tail vein, in several portions every 1-3 other weeks. An amount of the antigen is reportedly between 1 μg to 100 mg, normally preferably about 50 μg. In general, immunization is performed 2 to 7 times, and various methods are known to effect the immunization. Next, antibody-producing cells derived from spleen, etc. are fused to cells such as myeloma cells that can grow in vitro. The antibody-producing cells can be acquired from the spleen, etc. of mice, nude mice, rats, etc.

[0017] The fusion above can be carried out in accordance with the known method established by Koehler and Milstein

(Nature, 256, 495, 1975) using polyethylene glycol (PEG). The fusion may also be carried out through electrofusion using Sendai virus.

[0018] The hybridomas capable of producing the antibody, which can recognize human TRACP 5b, are selected from the fused cells described above, which selection proceeds as follows. After fusion, the resulting hybridomas are maintained in HAT medium and HT medium, cloned by limiting dilution and selected in the colonies produced from the maintained cells. When antibodies to human TRACP 5b are contained in the culture supernatants of the colonies produced from the fused cells plated on a 96-well plate, etc., the supernatants are placed on human TRACP 5b-immobilized assay plates. Following the reaction, clones capable of producing the monoclonal antibody can be screened by ELISA, which involves reacting a labeled secondary antibody such as anti-mouse-immunoglobulin HRP-labeled antibody. For labeling the antibody, a label including an enzyme such as alkaline phosphatase, a fluorescent material, a radioactive substance, etc. can be used,- in addition to HRP. For control, ELISA is simultaneously performed on the assay plates coated only with BSA as a blocking agent, thereby to screen human TRACP 5b-specific antibodies. In short, clones which are positive on the human TRACP 5b-coated plates and negative by ELISA using BSA can be screened.

[0019] In the hybridomas which produce monoclonal antibodies capable of recognizing human TRACP 5b, preferred examples of the hybridomas are hybridomas producing the monoclonal antibodies, which are reactive particularly with human TRACP 5b and are not cross-reactive with erythrocyte-, platelet-, neutrophil- and prostate-derived acid phosphatases. One of the examples is hybridoma Trk62 established by the present inventors.

[0020] For the monoclonal antibodies of the invention, monoclonal antibodies having a higher affinity to human TRACP 5b than to human TRACP 5a are particularly preferred and those showing a higher reactivity with human TRACP 5b by twice or more than the reactivity with human TRACP 5a in the detection system are more preferred, since the test results reflect bone resorption more clearly when they are used for clinical tests. An example of such hybridomas is hybridoma Trk62 established by the inventors. Hybridoma Trk62 has been internationally deposited under the Budapest Treaty on February 14, 2002, at National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, under the accession number of FERM BP-7890.

[0021] The hybridomas described above are cultured in medium conventionally used for cell culture, e.g., α-MEM, RPMI1640, ASF, S-clone, etc., to recover the monoclonal antibody from the culture supernatants. The monoclonal antibody may also be produced by the procedure which involves intraperitoneally injecting the cells into pristane-primed animals or nude mice of the hybridoma origin thereby to retain the ascites fluid, and collecting and purifying the retained ascites fluid to recover the monoclonal antibody.

[0022] For recovering the monoclonal antibody from the supernatants or ascites fluids described above, conventional methods are available. Examples of the conventional methods are salting out with ammonium sulfate, sodium sulfate, etc.; chromatography, ion exchange chromatography, affinity chromatography using protein A, protein G, etc.

[0023] The monoclonal antibody of the invention thus acquired is more highly reactive with TRACP 5b than with TRACP 5a, and more specific to TRACP 5b than to TRACP 5a. Specifically, the monoclonal antibody of the invention has a higher reactivity with TRACP 5b by twice or more than the reactivity with TRACP 5a. More specifically, the monoclonal antibody has a higher reactivity with TRACP 5b by twice or more than the reactivity with TRACP 5a, when TRACP 5a and 5b of an equal activity are reacted with the monoclonal antibody, respectively. Still more specifically, when TRACP 5a and TRACP 5b, which show the enzymatic activity of 10 U/L in the reaction at pH 5.7 (i.e., pH 5.7 at which TRACP 5a and TRACP 5b show the same enzymatic activity) in the presence of tartaric acid or a tartrate using p-nitrophenyl phosphate (pNPP) substrate, are reacted with the monoclonal antibody of the invention immobilized on a plate, respectively, and the respective enzymatic activities of TRACP 5a and TRACP 5b conjugated to the antibody are measured at pH 6.1 (i.e., optimal pH value of TRACP 5b) in the presence of tartaric acid or a tartrate using the above pNPP substrate, the monoclonal antibodies having a higher reactivity with TRACP 5b by twice or more, preferably 3 times or more, than the reactivity with TRACP 5a, are particularly preferred, as will be clearly indicated in EXAMPLE 1 (8) later described for specificity assay.

[0024] Moreover, as will also be clearly demonstrated in EXAMPLE 1 (8) described for specificity assay, the monoclonal antibody of the invention does not show any substantial cross-reactivity with erythrocyte-, platelet-, neutrophil- and prostate-derived acid phosphatases. Furthermore, the monoclonal antibody of the invention can recognize the steric structure of TRACP 5b retained in the native enzyme form, which will be clearly demonstrated in EXAMPLE 1 (7) for Western blotting and EXAMPLE 4.

[0025] The monoclonal antibody of the invention enables to specifically detect TRACP 5b in a sample with high sensitivity. Samples of interest include blood, serum, plasma, tissues such as bone, etc.

[0026] In the method for detection of TRACP 5b according to the invention, TRACP 5b in a sample can be detected by, for example, an immunoassay utilizing the determination of the enzymatic activity of TRACP 5b, a sandwich ELISA and an tissue immunostaining assay.

[0027] The immunoassay utilizing the determination of the enzymatic activity of TRACP 5b includes a method wherein TRACP in a sample such as serum is bound to the monoclonal antibody of the invention, the bound TRACP 5b is

reacted with an enzyme substrate of TRACP 5b such as p-nitrophenyl phosphate, and the enzymatic activity of the bound TRACP 5b is thus determined. More specifically, in such method, the sample containing TRACP 5b is added to the monoclonal antibody of the invention immobilized on a solid phase support, so that TRACP 5b in the sample is subjected to an antigen-antibody reaction and TRACP 5b is thus bound to the antibody immobilized on the solid phase support. Then, the solid phase support is washed to remove other components of the sample which have not been bound to the antibody. Thereafter, the enzyme substrate of TRACP 5b such as p-nitrophenyl phosphate or its salt is added to the reaction system, and the substrate is reacted with the TRACP 5b bound to the antibody. After the enzymatic reaction is terminated with a reaction terminating solution, a component such as p-nitrophenol resulted from the enzymatic reaction is determined by measuring the absorbance at wavelength of 390 nm to 450 nm, preferably 400 nm to 430 nm. The absorbance is the indicative of the enzymatic activity of TRACP 5b, and TRACP 5b in the sample is therefore determined.

[0028] As seen from the above, the antibody of the invention is preferably used as an antibody immobilized on a solid phase support. Any solid phase supports conventionally used in a solid phase immunoassay such as ELISA may be used in the method of the invention without limitation. The solid phase support may be made from polystyrene, polypropylene, polycarbonate, polyethylene, nylon, polymethacrylate and the like. The support may be in the form of plate or beads.

[0029] The antibody immobilized on the solid phase support may be prepared by binding directly or indirectly the antibody to the support through physical or chemical binding or affinity. The amount of the antibody sensitized is generally in a range of 1 ng to 100 mg/ml.

[0030] For carrying the method, a kit may be used containing a solid phase support, the monoclonal antibody of the invention and an enzyme substrate of TRACP 5b. In the kit, the solid phase support and the antibody may be provided separately from each other, and the antibody may be immobilized on the support just prior use. Alternatively, the antibody may have been immobilized on the support previously prior use. The kit may contain a washing solution for removing from the support other unbound components in the sample after TRACP 5b has been bound to the support. An amount of the antibody sensitized is generally in a range of 1 ng to 100 mg/ml. The washing solution includes Tris buffer containing surface active agents. The kit may preferably contain a reaction terminating solution including an aqueous alkaline solution such as aqueous sodium hydroxide and potassium hydroxide solutions. Further, the kit may, if necessary, contain a dilution solution for the sample including buffer solutions such as Tris buffer. The buffer solution may, if necessary, contain a chelating agent such as EDTA·2Na and an inorganic salt such as NaCl.

[0031] In the method of the invention, TRACP 5b in a sample is detected according to a sandwich ELISA using the monoclonal antibody of the invention. In the ELISA, an antibody other than the invention is used. Specifically, the antibody of the invention as a first antibody is immobilized on a solid phase support such as a plate, and then TRACP 5b in a sample such as serum is reacted with the immobilized antibody, followed by washing of the support. Thereafter, TRACP 5b bound to the support is reacted with a second antibody such as a biotinated other monoclonal antibody, polyclonal antibody or antiserum specific for TRACP 5b, followed by the reaction with peroxidase-labeled streptoavidine. Then, a peroxidase enzymatic reaction is carried out followed by a coloring reaction, whereby TRACP 5b in the sample is determined. As the other second antibody, there may be used the antibodies labeled with enzymes such as peroxidase and alkaline phosphatase. Otherwise, as the second antibody there may be used antibodies labeled with radioisotope, fluorescent compound, magnetic compound or colloid.

[0032] The sandwich ELISA may be carried out using a kit containing a solid phase support, a monoclonal antibody of the invention, labeled other antibody specific for TRACP 5b and components for detecting the labeled antibody. When the label is biotin, the components for detecting the labeled antibody may be peroxidase-labeled streptoavidine, tetramethylbenzidine as a substrate for the peroxidase and hydrogen peroxide. When the label is alkaline phophatase, the components may be reagents containing p-nitrophenyl phosphate. The kit may, if necessary, contain a washing solution.

[0033] In the invention, the presence of TRACP 5b in a sample can be detected by a tissue immunostaining assay using the monoclonal antibody of the invention. For example, a frozen section is prepared from, for example, human osteoclast cell tissue according to a conventional manner, and the monoclonal antibody of the invention is reacted therewith. The reaction product is then reacted with a second antibody labeled with, for example, an enzyme such as an alkaline phosphatase, followed by the coloring reaction for observation. In this fashion, the presence of TRACP 5b can be detected specifically.

[0034] Kits suitable for use in the detection method may include the monoclonal antibody as a first antibody, a labeled second antibody and reagents for staining the labeled second antibody. The reagents for staining the antibody may contain chromogenic substrates.

[0035] Hereinafter the invention will be described in more detail, with reference to the following preferred embodiments, but is not deemed to be limited only to these EXAMPLES.

EXAMPLE 1

Production of monoclonal antibody highly specific to osteoclast-derived tartrate-resistant acid phosphatase (TRACP 5b) and its properties

(1) Purification of TRACP 5b

[0036] After informed consent was obtained, 130 g of human femoral caput surgically dissected was frozen in liquid nitrogen followed by pulverizing with a hammer. The resulting powders were suspended in 200 mL of buffer solution (50 mM Tris-HCl, 0.3 M KCl, pH 7.5) containing a protease inhibitor, etc. and the suspension was homogenized by a ultrasonic homogenizer. After stirring at 4°C overnight, centrifugation was conducted at 10,000 rpm for 20 minutes. The supernatant was dialyzed to 10 mM Tris buffer (pH 8.2), and the dialysate was passed through CM-Sepharose Column (Sigma Inc.). The protein adsorbed was eluted on a linear gradient of the Tris buffer containing NaCl. The tartrate-resistant acid phosphatase activity was assayed using pNPP substrate, and fractions having a high activity were pooled. After condensation, the condensate was dialyzed to 20 mM Tris buffer, pH 7.2, containing 0.7 M NaCl and the dialysate was passed through Superdex 200 Column (Amersham Pharmacia Inc.). The tartrate-resistant acid phosphatase activity in the eluate was assayed as above and the active fractions were pooled. The pooled fractions were applied to HiTrap Heparin HP Column (Amersham Pharmacia, Inc.) to elute the adsorbed protein on a linear gradient of the 20 mM Tris buffer, pH 7.4, containing NaCl. The highly active tartrate-resistant acid phosphatase fractions were pooled and concentrated to give 0.4 mg of purified TRACP 5b. The amount of protein was determined by A_{280}, and after SDS-PAGE (TIFCO) followed by silver staining, the protein was purified until it showed a single band having a molecular weight of about 35,000. Thus, the protein was identified to be TRACP 5b (FIG. 1). The enzyme purified to a single band was used as purified TRACP 5b immunogen.

(2) Immunization of mice with purified human TRACP 5b

[0037] Purified human TRACP 5b was diluted to 250 μg/ml with 50 mM citrate buffer (pH 5.5) and an aliquot of 25 μg (100 μl) was taken and thoroughly mixed with 100 μl of Freund's complete adjuvant (Wako Pure Chemical Industries, Ltd.) until the mixture was emulsified. The emulsified suspension prepared was intraperitoneally injected with Balb/c female mice of 6 weeks old (CLEA Japan Inc.) under diethyl ether anesthesia. After 2 weeks, an equal amount of TRACP 5b (25 μg/ml) was mixed with Freund's incomplete adjuvant (Wako Pure Chemical Industries, Ltd.) until the mixture became emulsified suspension, in the same manner as with Freund's complete adjuvant, followed by immunization to mice. Then the same procedures were repeated at biweekly intervals. For the fourth booster, 25 μg/ml of TRACP 5b in 50 mM citrate buffer (pH 5.5) was given intravenously through the mice tail for final immunization.

(3) Establishment of hybridomas

[0038] Three days after the final immunization, the spleen surgically dissected from the TRACP 5b-immunized mice under diethyl ether anesthesia was aseptically dispersed to prepare splenocytes. The cell fusion was carried out according to the method of Kohler and Milstein (Nature, 256: 495, 1975), and the splenocytes were fused to myeloma cells P3-X63-Ag8-U1 (P3U1) using polyethylene glycol (PEG 4000) (Merck, Inc.). The fusion of TRACP 5b was made between 2 x 10^7 of myeloma cells P3-X63-Ag8-U1 (P3U1) and 8 x 10^7 of the splenocytes, indicating that the fusion ratio of myeloma cells to splenocytes was approximately 4 : 1. The fused cells were dispersed in 10% FCS (INVITROGEN, Inc.)-containing α-MEM (IRVINE, Inc.) HAT (Cosmobio Co., Ltd.) medium, dispensing the suspension onto 96-well microtiter culture plates (Sumitomo Bakelite Co., Ltd.) and culturing at 37°C in 5% CO_2.

(4) Screening of colonies

[0039] After approximately 2 weeks, it was confirmed that colonies were grown and ready for screening. The screening protocols performed are described below.

[0040] To prepare plates for screening, TRACP 5b purified in (1) above was dissolved in 50 mM citrate buffer. The solution was dispensed into 96-well microtiter plates (Nunc, Inc.) in an amount of 0.5 μg/100 μl/well. The plates were allowed to stand at 4°C for 2 nights followed by washing 3 times with Tris buffer containing 0.05% Tween 20 (Wako Pure Chemical Industries, Ltd.). Then a 200 μl aliquot of 1.5% BSA (SIGMA, Inc.) solution was dispensed on each well to block non-specific reactions, which was further allowed to stand at 4°C overnight. After the plates obtained were washed 3 times with Tris buffer containing 0.05% Tween 20, 100 μl of the culture supernatant of hybridoma obtained in (3) above was reacted therewith. Further washing was followed by addition of a secondary antibody, i.e., HRP-conjugated anti-mouse immunoglobulin antibody (Zymed, Inc.) to react them. After washing, 100 μl of a citrate solution

containing 3 mg/ml of o-phenylenediamine (OPD) (Nakarai Co., Ltd.) as a chromogenic substrate for HRP was added to the mixture and allowed to stand for a given period of time for color formation. To terminate the reaction 100 µl of 1N sulfuric acid (Wako Pure Chemical Industries, Ltd.) was further added to the mixture, and absorbance was measured at a measuring wavelength of 492 nm. The clones that turned positive as described above were recloned by limiting dilution and the supernatant was monitored again.

(5) Identification of antibody

[0041] By confirming the reactivity of the clones with purified TRACP 5b by ELISA, clones Trk62 and Trk49 reactive with the purified TRACP 5b-coated plates were acquired. The monoclonal antibody Trk62 produced by clone Trk62 showed a strong reactivity with the purified TRACP 5b, whereas monoclonal antibody Trk62 did not react with the BSA-coated plates for vehicle. On the other hand, the monoclonal antibody Trk49 produced by clone Trk49 showed a weak reactivity with the purified TRACP 5b. The above antibodies Trk62 and Trk49 were assayed on a monoclonal antibody typing kit (Amersham Pharmacia, Inc.) and found to have the following properties.

Class	Subclass	Light Chain
IgG	IgG1	k

(6) Production and purification of monoclonal antibody

[0042] Hybridoma Trk62 obtained in (4) above was intraperitoneally administered in a dose of 1 x 10^7 cells to Balb/c mice (CLEA Japan, Inc.) (10 weeks old, female) 2 weeks after priming with 0.5 ml of pristane (Aldrich, Inc.). The ascites fluids retained in the ascites cavities of mice were surgically collected under diethyl ether anesthesis. The collected ascites fluid was used as a sample and serially diluted. The ascites fluid dilution was monitored by ELISA in the same manner as used in the procedures for screening of the colonies in (4) described and found that the dilution contained a high level of the monoclonal antibody. This ascites fluid was treated with 40% ammonium sulfate and dialyzed to PBS. The dialysate was purified through Protein G Column (Amersham Pharmacia, Inc.) and confirmed on SDS-PAGE. Thus, the monoclonal antibody Trk62 was found to have a single band at a molecular weight of about 150,000 under nonreducing conditions and and 2 bands at molecular weights of about 50,000 and about 25,000 under methanol-reducing conditions. The purified monoclonal antibody was about 15 mg per mouse, which was sufficient for industrial application. Similarly, the monoclonal antibody Trk49 was obtained from the hybridoma Trk49. The hybridoma Trk49 has been internationally deposited under the Budapest Treaty on November 27, 2002, at National Institute of Advanced Industrial Science and Technology, International Patent Organism Repositary, under the accession number of FERM BP-8249.

(7) Western blotting

[0043] Based on the results of western blotting, etc., it was found that monoclonal antibody Trk62 did not recognize SDS-treated TRACP 5 but recognized native TRACP 5, which is described below in detail.

[0044] After obtaining informed consent, TRACP was purified from human cord blood in accordance with the method already known (Clin. Chem., 24:7, 1105-1108, 1978) and used as samples for western blotting. Upon the purification, human TRACP was separated into 2 isoforms, TRACP 5a and TRACP 5b. TRACP 5a and TRACP 5b were identified by the disc electrophoresis (Clin. Chem., 24:2, 309-312, 1978 (FIG. 2) and by the activity assay using a fluoride as an inhibitor (Clin. Chem., 46:4, 469-473, 2000). Separately, human recombinant TRACP (Baculoviral rhTRACP) was produced using insect cells by modifying the method of Hyman, et al. (J. Biol. Chem., 269, 1294-1300, 1994) already known in the art, purified and then used as one of antigens (Fractions 26, 27 and 28 shown in FIG. 3 were pooled and used). From each of the three enzyme proteins (human TRACP 5a, TRACP 5b and Baculoviral rhTRACP), 2 µg was taken and subjected to SDS-PAGE under nonreducing conditions. Monitoring of the Trk62 reactivity by western blotting reveals that Trk62 did not react with any of human TRACP 5a, TRACP 5b and Baculoviral rhTRACP. However, anti-TRACP monoclonal antibody 9C5 (Zymed, Inc.) (Hybridoma, 16: 175-182, 1997; BioTec. Histochem., 73:316-324, 1998) used as a positive control reacted with 33 kDa human TRACP 5a, TRACP 5b and Baculoviral rhTRACP and with a small fragment of 16 kDa, which fragment was observed in all three antigens (FIG. 4). It is already known that anti-TRACP monoclonal antibody 9C5 reacts with the small fragment of 16 kDa modified by thermal degeneration (Hybridoma, 16: 175-182, 1997 Biotech. Histochem., 73: 316-324, 1998).

[0045] The results reveal that surfactant SDS changed the steric structures of human TRACP 5a, TRACP 5b and Baculoviral rhTRACP and because of the absence of detectable steric structures, the monoclonal antibody Trk62 of the invention lost its reactivity with human TRACP 5a, TRACP 5b and Baculoviral rhTRACP. This is because Trk62 is

the monoclonal antibody which recognizes the steric structures of human TRACP 5a, TRACP 5b and Baculoviral rhTRACP. The results also reveal that monoclonal antibody Trk62 recognizes the native structure of TRACP.

(8) specificity assay

[0046] The monoclonal antibody Trk62 was tested for specificity by the following procedures. The assay method comprises steps (i) through (v) below.

(i) After anti-mouse immunoglobulin (DAKO Japan) was dispensed to the microtiter plates (Nunc, Inc.) used for the screening of colonies in (4) above in 1 μg/100 μl/well. The plates were allowed to stand at 4°C for 2 nights. Then, the plates were washed 3 times with 0.05% Tween 20-containing Tris (SIGMA, Inc.) buffer and 200 μl aliquot of 1.5% BSA (SIGMA, Inc.) solution was dispensed into the plates to block non-specific reactions. The plates were further allowed to stand at 4°C overnight.

(ii) The plates thus completed were washed 3 times with 0.05% Tween 20-containing Tris buffer and reacted at room temperature for an hour for comparison with 400 ng/100 μl/well of monoclonal antibody Trk62 of the invention and with monoclonal antibody O1A (attached to BoneTRAP Assay Kit available from Suomen Bioanalytiikka Oy; monoclonal antibody described in WO 99/50662 and P2002-510050A). The plates were further washed 3 times with 0.05% Tween 20-containing Tris buffer.

(iii) Next, the monoclonal antibodies Trk62-and O1A-reacted plates were reacted with various acid phosphatases. The acid phosphatases used as sample were purified human TRACP 5a and Baculoviral rhTRACP used for the western blotting in (7) above, and TRACP 5b used for sensitization, erythrocyte extract, platelet extract, neutrophil extract and prostate-derived acid phosphatase (PAP) (SIGMA, Inc.). In specimens containing these ACP isoforms, the enzymatic activity of TRACP was all adjusted to 10 U/L (activity level measured in 8 mM pNPP substrate solution, 0.1 M sodium acetate and 40 mM sodium tartrate, pH 5.7), and 100 μl aliquot was added onto the antibody-bound plates.

(iv) After reacting at room temperature for an hour, the plates were washed 3 times with 0.05% Tween 20-containing Tris buffer, and 100 μl of the substrate solution (8 mM pNPP, 100 mM sodium acetate and 40 mM sodium tartrate, pH 6.1) was added thereto. The mixture was allowed to stand for an hour at 37°C for color formation. Absorbance at 405 nm was measured and the amounts of various ACP isoforms reacted with monoclonal antibodies Trk62 and O1A, respectively, were determined. The measurement data were calculated by subtracting the blank absorbance using saline alone as a specimen from the absorbance of each specimen. The measurement data obtained are shown in TABLE 1.

TABLE 1:

Amount of monoclonal antibody reacted with ACP isoforms (absorbance at 405 nm absorbance obtained by subtracting the blank absorbance)		
ACP Isoforms	Monoclonal antibody O1A for Comparison	Monoclonal antibody Trk62 of Invention
Neutrophil	0.003	0.002
Platelet	0.002	0.001
Erythrocyte	0.001	0.002
Prostate	0.002	0.001
Baculoviral rhTRACP	0.476 (75%)	0.486 (70%)
TRACP 5a	0.413 (65%)	0.222 (32%)
TRACP 5b	0.636 (100%)	0.694 (100%)
TRACP 5b/TRACP 5a	1.54	3.13

Percentage within parenthesis indicates the reactivity when absorbance of TRACP 5b was made 100%.

[0047] The results shown in TABLE 1 reveal that monoclonal antibodies O1A and Trk62 reacted only with human TRACP 5b, TRACP 5a and Baculoviral rhTRACP but did not show any cross-reactivity with other ACP isoforms, though the reaction percentage was different. That is, when the reactivity with TRACP 5b was made 100%, the reactivity of two antibodies with Baculoviral rhTRACP was about 70%. However, the reactivity of TRACP 5a with monoclonal an-

tibody O1A was 65%, whereas the reactivity with monoclonal antibody Trk62 was only 32%. This means that monoclonal antibody Trk62 has a higher affinity especially to TRACP 5b. That is, the ratio in the reactivity of TRACP 5b to TRACP 5a (TRACP 5b/TRACP 5a) was 1.54 for monoclonal antibody O1A and 3.13 for monoclonal antibody Trk62, indicating that monoclonal antibody Trk62 provides a higher specificity to TRACP 5b by about twice, as compared to monoclonal antibody O1A.

[0048] Based on the foregoing results, the monoclonal antibody Trk62 of the invention has the following properties.

[0049] That is, each of TRACP 5a and TRACP 5b, which show the enzymatic activity of 10 U/L when reacted at pH 5.7 in the presence of sodium tartrate using p-nitrophenyl phosphate (pNPP) substrate, is reacted with the monoclonal antibody of the invention immobilized to the plates, and the enzymatic activity of each of TRACP 5a and TRACP 5b bound to the plates is assayed at pH 6.1 using the pNPP substrate described above. The reactivity with TRACP 5b is higher by 3.13 times than the reactivity with TRACP 5a.

EXAMPLE 2

Immunoassay utilizing the determination of the enzymatic activity of TRACP 5b and using monoclonal antibody Trk62 for clinical sampler

[0050] In order to ascertain the clinical significance of monoclonal antibody Trk62 in association with bone resorption-related diseases, sera collected from the patients who received hormone replacement therapy (HRT) were used as samples to determine the TRACP 5b level in the samples.

(1) Method

[0051] The assay for TRACP 5b using monoclonal antibody Trk62 was carried out by the same procedures as used for the specificity assay in EXAMPLE 1 (8), provided that the assay data was calculated by measuring recombinant TRACP attached to the BoneTRAP Assay Kit (Suomen Bioanalytiikka Oy) for standard and converting the absorbance into U/L enzymatic activity unit based on the calibration curve thus obtained. For comparison, the the biochemical assay for the total TRACP activity (Clin. Chem., 44: 221-225, 1998) and ELISA for TRACP 5b were carried out using BoneTRAP Assay without any modification. Samples were collected from the patients who gave informed consent, and stored at -80°C until they were provided for testing. Intervals before and after HRT were 7.4 months in average.

(2) Results

[0052] The results obtained are shown in TABLE 2.

TABLE 2:

Assay of TRACP 5b in clinical samples						
	Comparison Total TRACE by biochemical assay TRACP 5b (IU/L) (%)		Comparison Monoclonall antibody O1A TRACP 5b (U/L) (%)		This Invention Monoclona antibody Trk62 (U/L) (%)	
No. 1: Before HRT	19.8		6.9		1.4	
After HRT	17.6	88.9%	2.6	37.7%	0.5	35.7%
No. 2: Before HRT	28.2		9.4		2.4	
After HRT	21.0	74.5%	6.1	64.9%	1.2	50.0%
No. 3: Before HRT	17.7		4.4		1.0	
After HRT	14.6	82.5%	3.4	77.3%	0.5	50.0%
No. 4: Before HRT	2.6		6.5		1.5	
After HRT	19.5	73.3%	4.6	70.8%	0.7	46.7%

TABLE 2: (continued)

Assay of TRACP 5b in clinical samples						
	Comparison Total TRACE by biochemical assay TRACP 5b (IU/L) (%)		Comparison antibody O1A1 ('		This Invention antibody Trk	
No. 5: Before HRT	22.3		5.9		0.8	
After HRT	19.5	87.4%	3.5	59.3%	0.5	62.5%
No. 6: Before HRT	24.5		7.6		1.1	
After HRT	17.3	70.6%	4.1	53.9%	0.2	18.2%
No. 7: Before HRT	22.9		5.6		1.0	
After HRT	13.4	58.5%	1.5	26.8%	0.0	0.0%
No. 8: Before HRT	21.0		6.3		0.9	
After HRT	15.8	75.2%	3.3	52.4%	0.2	22.2%
No. 9: Before HRT	24.7		6.1		1.4	
After HRT	21.6	87.4%	4.2	68.9%	0.8	57.1%
No. 10: Before HRT	21.9		6.1		1.0	
After HRT	16.1	73.5%	3.1	50.8%	0.1	10.0%
No. 11: Before HRT	24.3		7.7		0.8	
After HRT	15.9	65.4%	3.9	50.6%	0.5	62.5%
No. 12: Before HRT	21.0		4.0		0.3	
After HRT	16.3	77.6%	2.5	62.5%	0.0	0.0%
No. 13: Before HRT	23.3		6.5		1.4	
After HRT	20.1	86.3%	3.7	56.9%	0.3	21.4%
Average ratio in post-therapy		77.0%		56.4%		33.6%

[0053] Ideal agents for clinical tests are those giving test data that decrease after HRT dynamically as compared to the data in the pre-therapy group. Then, therapeutic effects can be assessed precisely. Therefore, when the test data obtained in the post-therapy are the smallest possible value as compared to the data before therapy, such agents are suitable for use in clinical tests. Reviewing the results shown in TABLE 2, the test data in the post-therapy group for assessing the therapeutic effects by ELISA using monoclonal antibody Trk62 acquired in EXAMPLE 1 indicated 33.5% in average based on the data before therapy, indicating a very large rate of change. These results proved more useful than those for comparison, i.e., the biochemical total TRACP assay indicating 77.0% and the BoneTRAP Assay for measuring TRACP 5b indicating 56.4%.

[0054] For comparison, TRACP 5b in the same samples was also assayed using conventional bone metabolism markers. The results were 80.5% for B-ALP (Osteolink [BAP], which measures serum bone-specific alkaline phosphatase by ELISA), 57.5% for NTx (Osteomark NTx, which measures urinary N-telopeptide of type I collagen degradation by ELISA), 68.5% for Pyr (total pyridinoline in urine on HPLC), 52.8% for D-Pyr (total deoxypyridinoline in urine on HPLC) and 77.8% for D-Pyr (Osteolink [DPD], which measures free deoxypyridinoline in urine by ELISA).

[0055] The results above reveal that TRACP 5b using monoclonal antibody Trk62 of the invention provides a larger rate of change before and after HRT than any one of the tested conventional markers of bone metabolism and is very useful from a clinical aspect.

EXAMPLE 3

Immunoassay of TRACP 5b in serum from adults and infants at pH of 5.65 using monoclonal antibody Trk62

[0056] The level of TRACP 5b in serum from adults having stable bone resorbing ability and infants showing vigorous bone resorption was assayed by using the monoclonal antibody of the invention.

(1) Method

[0057] The assay was carried out by the same manner as used in EXAMPLE 2 except that only the pH of substrate pNPP was changed from 6.1 to 5.65. For comparison, TRACP 5b was determined by BoneTRAP Assay using the above monoclonal antibody 01A. Two sera each collected from the adults and children who gave informed consent were used as samples. It is said that TRACP 5b responsible for bone resorption provides a higher value in the infant serum because of vigorous bone metabolism.

(2) Results

[0058] The results obtained are shown in TABLE 3.

TABLE 3:

Assay of TRACP 5b in serum from infant and adult (absorbance at 405 nm: value obtained by subtracting the blank value)		
	Comparison Monoclonal antibody O1A	This Invention Monoclonal antibody Trk62
Child sample #1	0.947	0.379
Child sample #2	0.552	0.180
Mean value in child samples	0.750	0.280
Adult sample #1	0.588	0.069
Adult sample #2	0.623	0.057
Mean value in Adult samples	0.606	0.063
Ratio of the child group to the adult group	1.24	4.44

[0059] As noted from TABLE 3, the ratio in assay value for TRACP 5b of the child group to the adult group is 4.44 times when monoclonal antibody Trk62 was used, and is larger than 1.24 times assayed on BoneTRAP Assay Kit. Further in the assay at pH of 5.65, bone absorption is reflected on TRACP 5b with a much better sensitivity, than in the comparison, wherein the assay was made around the optimum pH of 6.1 for TRACP 5b. For information, it is said that infant samples give higher assay values of TRACP 5b reflecting bone absorption, since bone metabolism is vigorous in infant samples.

EXAMPLE 4

Sandwich ELISA of TRACP 5b in serum samples from normal adults and normal infants

[0060] TRACP 5b in each of the samples was quantitatively detected according to a sandwich ELISA using the monoclonal antibody Trk62 of the invention and the monoclonal antibody Trk49 which is outside the invention.

(1) Method

[0061] The monoclonal antibody Trk62 was dispensed to solid phase plates in 1 μg/μL, and allowed to stand at 4°C for 2 days, whereby the antibody-immobilized plates were prepared, followed by blocking. After the plates were washed three times with 0.05% Tween 20-containing Tris buffer, 50 μl of the serum sample and 500 μl of citrate buffer were separately added to the plates, and allowed to stand at room temperature for one hour, whereby TRACP 5b in the samples was bound to the antibody immobilized on the plates. After washing, to the remained TRACP 5b bound to the

antibody immobilized on the plate was added 100 μl of a solution of a horseradish peroxidase-conjugated antibody Trk49 as the second antibody, and reacted with the remained TRACP 5b on the plate to form sandwich complexes. After three times washing, 3 mg/mL of OPD (NAKALAI, Inc.) as a chromogenic substrate for the horseradish peroxidase. After a predetermined period, 1N sulfuric acid as a stop solution was added to terminate the reaction. Then, the absorbance at wavelength of 490 nm was measured for the samples. Thus, the measured absorbance value (OD) was converted to the concentration of TRACP 5b in the samples according to a standard curve previously prepared using purified TRACP 5b.

(b) Results

[0062] TABLE 4 shows results obtained from the sandwich ELISA for six serum samples of normal adults and six serum samples of normal children. The average concentration of TRACP 5b for six serum samples of normal adults was 9.92 ng/mL, whereas the average was 34.00 ng/mL for normal children which were active in bone absorption.

[0063] Those results indicate that the sandwich ELISA according to the invention accurately and correctly reflects the active bone absorption.

TABLE 4:

Results from Sandwich ELISA for TRACP 5b	
Normal adults (ng/mL)	Normal children (ng/mL)
7.66	23.60
9.74	17.31
10.00	45.55
11.16	37.46
9.24	53.39
11.71	26.70
average 9.92	average 34.00

EXAMPLE 5

Tissue immunostaining assay of TRACP 5b using monoclonal antibody Trk62

(1) Method

[0064] Frozen human osteoclastoma tumor tissue thinly sliced at a thickness of 2 μm was treated with acetone at -20°C to fix the tissue. After washing, the tissue sections were treated with 3% hydrogen peroxide aqueous solution for endogenous peroxidase treatment. Paraffin-embedded sections of the same sample were cut on a microtome and treated 3 times in xylene each for 5 minutes for complete deparaffinization. The sections were then rehydrated by passing them through a graded alcohol solution from 100% to 50% ethanol descending by 10% in 6 steps and microwaved with 50% citrate buffer (pH 6.0) for infiltration. Thereafter, the frozen sections and the paraffin sections were both reacted in the same way. That is, after blocking with 50 mM citrate buffer (pH 6.0) containing 5% BSA, the sections were washed and reacted at room temperature for 2 hours with monoclonal antibody Trk62, which was purified from the ascites fluid and diluted to 10 μg/ml in 50 mM citrate buffer (pH 6.0) containing 5% BSA. For positive control, anti-TRAP antibody 9C5 (ZYMED, Inc.) used in the western blotting test was reacted. After washing was performed 5 times, the sections were incubated for another hour at room temperature with a secondary antibody using ENVISION Kit (DAKO Japan, Inc.). The sections were then washed further 5 times and stained with DAB chromogen kit(DAKO Japan, Inc.). Washing was followed by microscopic observation.

(2) Results

[0065] The results of microscopic observation are shown in FIG. 5.

[0066] In the monoclonal antibody Trk62 group, cytoplasm alone of the osteoclasts was selectively stained in the frozen sections. In the paraffin sections, no response was observed at all. In the anti-TRAP antibody 9C5 group, no detectable response was observed in the frozen sections and in the paraffin sections, osteoclasts were stained.

[0067] These results proved that monoclonal antibody Trk62 can recognize TRACP 5b retaining the steric structure.

This is also confirmed by the fact that the monoclonal antibody was reactive only with the frozen sections that retained the steric structure of enzyme. Moreover, the results proved that quick staining of samples provided for operation can be perioperatively made using the monoclonal antibody of the invention, which could assist to make histological diagnosis.

[0068] As described above in detail, the monoclonal antibody of the invention provides a higher reactivity with TRACP 5b than with TRACP 5a and a higher specificity to TRACP 5b. Therefore, TRACP 5b in a sample can be specifically detected by using the monoclonal antibody of the invention. The monoclonal antibody of the invention can specifically detect TRACP 5b as a marker for bone resorption with high sensitivity, and is thus extremely useful as an indicator of bone-associated disorders in clinical tests, etc.

Claims

1. A monoclonal antibody to tartrate-resistant acid phosphatase 5b (TRACP 5b, also known as osteoclast-derived tartrate-resistant acid phosphatase), which has a higher reactivity with TRACP 5b than tartrate-resistant acid phosphatase 5a (TRACP 5a) and a higher specificity to TRACP 5b.

2. The monoclonal antibody according to claim 1, wherein the reactivity with TRACP 5b is at least twice that of the reactivity with TRACP 5a.

3. The monoclonal antibody according to claim 1 or 2, wherein the reactivity with TRACP 5b is at least twice that of the reactivity with TRACP 5a, when TRACP 5a and TRACP 5b having an equal activity are reacted with the monoclonal antibody, respectively.

4. The monoclonal antibody according to any of claims 1 to 3, wherein the reactivity with TRACP 5b is at least twice that of the reactivity with TRACP 5a, when TRACP 5a and TRACP 5b, which show the enzymatic activity of 10 U/L in the reaction at pH 5.7 in the presence of tartaric acid or a tartrate together with p-nitrophenyl phosphate (pNPP) substrate, are reacted with the monoclonal antibody, respectively, and the respective enzymatic activities of TRACP 5a and TRACP 5b are measured at pH 6.1 in the presence of tartaric acid or a tartrate together with pNPP substrate.

5. The monoclonal antibody according to any of claims 1 to 4, which does not show any substantial cross-reactivity with erythrocyte-, platelet-, neutrophil-and prostate-derived acid phosphatases.

6. The monoclonal antibody according to any of claims 1 to 5, which can recognize the steric structure of TRACP 5b retained in the native enzyme form.

7. A hybridoma capable of producing the monoclonal antibody according to any of claims 1 to 6.

8. The hybridoma according to claim 7, which has been deposited at National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, under the accession number of FERM BP-7890.

9. A method for detection of TRACP 5b, which comprises detecting TRACP 5b in a sample by an immunoassay using the monoclonal antibody of any of claims 1 to 6.

10. The method for detection according to claim 9, which comprises binding TRACP 5b in a sample to the monoclonal antibody of any of claims 1 to 6 and determining the enzyme activity of TRACP 5b bound.

11. The method for detection according to claim 9, which comprises detecting TRACP 5b in a sample by a sandwich ELISA using the monoclonal antibody of any of claims 1 to 6.

12. The method for detection according to claim 9, which comprises detecting the presence of TRACP 5b in a sample by a tissue immunostaining assay using the monoclonal antibody of any of claims 1 to 6.

13. The method for detection according to claim 9, wherein the monoclonal antibody of any of claims 1 to 6 is used as a marker for bone resorption in a clinical test of bone-associated disorders.

14. A kit for use in the detection of TRACP 5b comprising the monoclonal antibody of any of claims 1 to 6.

DOCID: <EP_____1359161A2_I_>

15. The kit according to claim 14, which is for use in the method of claim 10 and comprises a solid phase support, the monoclonal antibody of any of claims 1 to 6 and an enzyme substrate of TRACP 5b.

16. The kit according to claim 14, which is for use in the method of claim 11 and comprises a solid phase support, the monoclonal antibody of any of claims 1 to 6, a labeled antibody specific for TRACP 5b other than that of claim 1 and a component for detecting the labeled antibody.

17. The kit according to claim 14, which is for use in the method of claim 12 and comprises the monoclonal antibody of any of claims 1 to 6 as a first antibody, a labeled antibody as a second antibody and reagents for staining the labeled second antibody.

FIG. 1

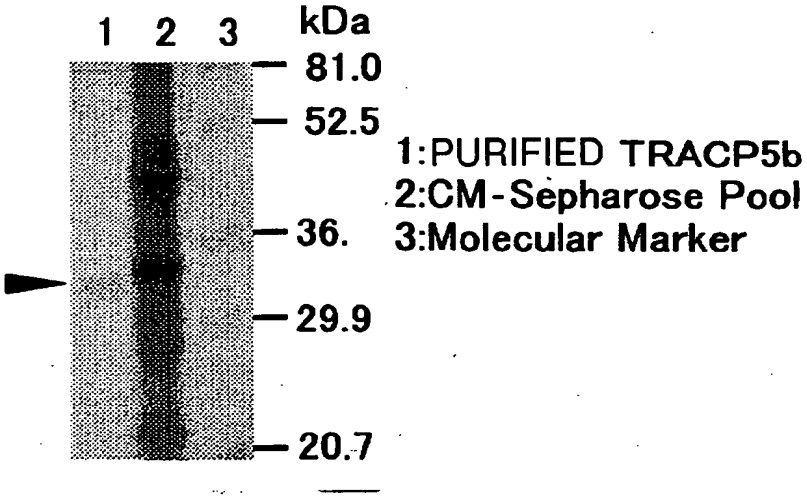
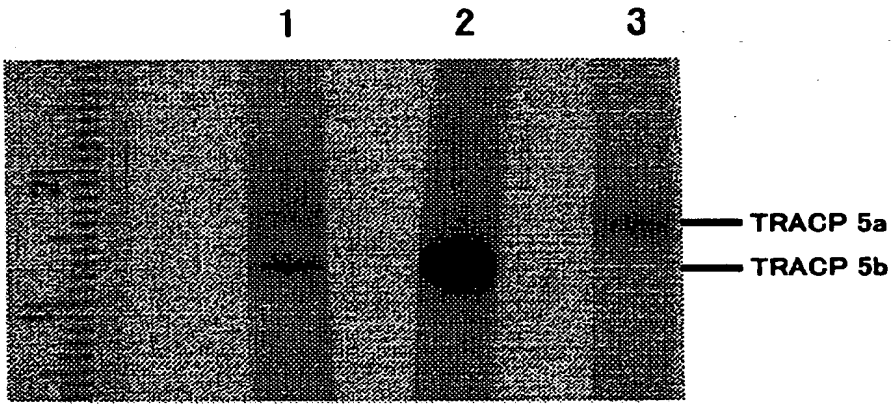
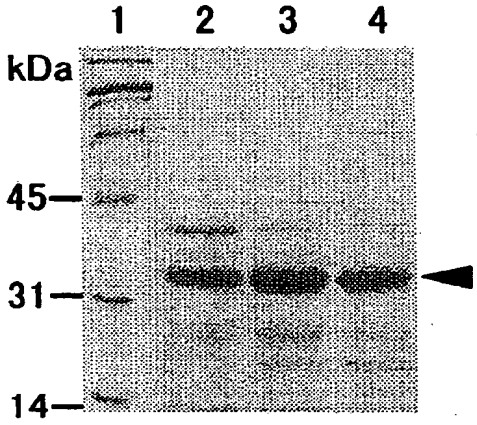


FIG. 2



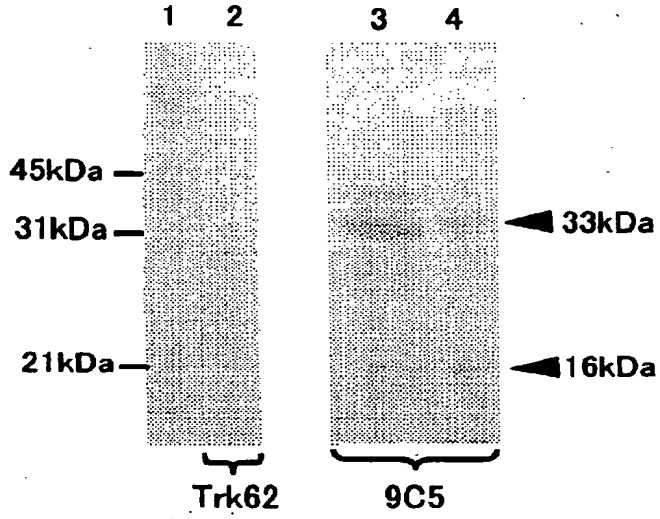
1: HUMAN SERUM
2: PURIFIED HUMAN TRACP 5b
3: PURIFIED HUMAN TRACP 5a

FIG. 3



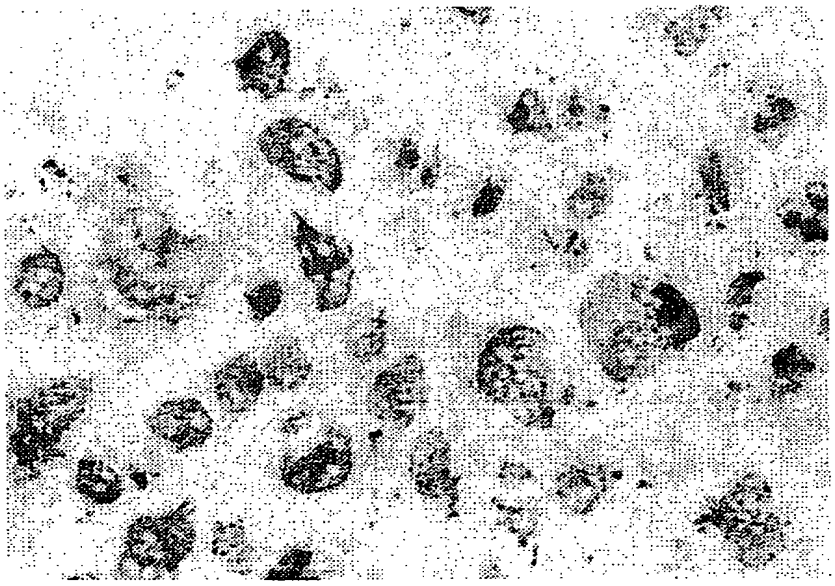
1 : Molecular Marker
2 : Fraction 26 (36.01KU/L)
3 : Fraction 27 (44.80KU/L)
4 : Fraction 28 (35.24KU/L)

FIG. 4



1: Molecular Marker
2: PURIFIED HUMAN TRACP 5b
3: Baculoviral rhTRACP
4: PURIFIED HUMAN TRACP 5b

FIG. 5



Trk62

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 359 161 A3

(12) EUROPEAN PATENT APPLICATION

(88) Date of publication A3:
21.01.2004 Bulletin 2004/04

(43) Date of publication A2:
05.11.2003 Bulletin 2003/45

(21) Application number: **03008868.6**

(22) Date of filing: **29.04.2003**

(51) Int Cl.[7]: **C07K 16/40**, C12N 5/20, G01N 33/577

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LI LU MC NL PT RO SE SI SK TR
Designated Extension States:
AL LT LV MK

(30) Priority: **30.04.2002 JP 2002128312**

(71) Applicant: **NITTO BOSEKI CO., LTD.**
Fukushima-shi (JP)

(72) Inventors:
- **Ohashi, Tatsuya, Baiokemikaru Kenkyusho**
  Fukuhara, Koriyama-shi, Fukushima (JP)
- **Miura, Toshihide, Baiokemikaru Kenkyusho**
  Fukuhara, Koriyama-shi, Fukushima (JP)
- **Igarashi, Yoshihiko,**
  c/o Dokkyo Univ.Sch.Medicine
  Mibumachi, Shimotsuga-gun, Tochigi-ken (JP)
- **Sasagawa, Kumiko, Baiokemikaru Kenkyusho**
  Fukuhara, Koriyama-shi, Fukushima (JP)
- **Katayama, Katsuhiro, c/o Nitto Boseki Co., Ltd.**
  Koriyama-shi, Fukushima (JP)

(74) Representative: **HOFFMANN - EITLE**
Patent- und Rechtsanwälte
Arabellastrasse 4
81925 München (DE)

(54) Monoclonal antibody specific to tartrate-resistant acid phosphatase 5b and use thereof

(57) Monocolonal antibodies having a higher reactivity with tartrate-resistant acid phosphatase 5b (TRACP 5b) than tartrate-resistant acid phosphatase 5a (TRACP 5a) and having a higher specificity to TRACP 5b can be obtained by cell fusion using as antigens TRACP 5b purified from human osteoclasts. By using the monoclonal antibody, TRACP 5b in a sample can be detected specifically with a high sensitivity.

EP 1 359 161 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 03 00 8868

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	J. HALLEEN ET AL.: "Tartrate-resistant acid phosphatase 5b: a novel serum marker of bone resorption." JOURNAL OF BONE AND MINERAL RESEARCH, vol. 15, no. 7, July 2000 (2000-07), pages 1337-1345, XP008024789 New York, NY, USA * abstract * * figure 1 * ---	1-6,9-13	C07K16/40 C12N5/20 G01N33/577
X	WO 99 50662 A (J. HALLEEN ET AL.) 7 October 1999 (1999-10-07) * the whole document * ---	7-17	
A	J. HALLEEN ET AL.: "Characterization of serum tartrate-resistant acid phosphatase and development of a direct two-site immunoassay." JOURNAL OF BONE AND MINERAL RESEARCH, vol. 13, 1998, pages 683-687, XP001029319 New York, NY, USA * the whole document * -----	11,16	

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

C07K
C12N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	24 November 2003	Nooij, F

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 03 00 8868

This annex lists the patent family membersrelating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

24-11-2003

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9950662 A	07-10-1999	FI 980748 A	02-10-1999
		AU 748663 B2	06-06-2002
		AU 3149199 A	18-10-1999
		CN 1295667 T	16-05-2001
		EP 1068533 A2	17-01-2001
		WO 9950662 A2	07-10-1999
		JP 2002510050 T	02-04-2002

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82